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Requested Patent: WO9318137A1

Title:

CULTURING OF HEMATOPOIETIC STEM CELLS AND THEIR GENETIC
ENGINEERING ;

Abstracted Patent: WO9318137 ;

Publication Date: 1993-09-16 ;

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Applicant(s): SYSTEMIX INC (US) ;

Application Number: WO1993US01852 19930303 ;

Priority Number(s): US19920846782 19920304 ;

IPC Classification: C12N5/06; C12N15/64 ;

Equivalents:

AU4369097, AU680406, CA2131368, EP0631618 (WO9318137), A4, JP7504331T ;

ABSTRACT:

Human hematopoietic stem cells may be grown in longterm culture, particularly co-cultures with an appropriate stromal cell line, particularly in the presence of leukemia inhibitory factor, by itself or in conjunction with other factors. The cells are found to retain their function as demonstrated by an in vivo T-cell assay and their ability to form colonies of other lineages in methylcellulose. Transfection of the cells may be achieved with a virus, where improved results are obtained in the presence of leukemia inhibitory factor and optionally other hematopoietic factors.

*medice changes
every 5-7 days*



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 5/06, 15/64	A1	(11) International Publication Number: WO 93/18137 (43) International Publication Date: 16 September 1993 (16.09.93)
(21) International Application Number: PCT/US93/01852 (22) International Filing Date: 3 March 1993 (03.03.93) (30) Priority data: 07/846,782 4 March 1992 (04.03.92) US (71) Applicant: SYSTEMIX, INC. [US/US]; 3155 Porter Drive, Palo Alto, CA 94304 (US). (72) Inventor: SHIH, Chu-Chih ; 3174 Greer Road, Palo Alto, CA 94303 (US). (74) Agents: ROWLAND, Bertram, I. et al.; Flehr, Hohbach, Test, Albritton & Herbert, 4 Embarcadero Center, Suite 3400, San Francisco, CA 94111-4187 (US).		(81) Designated States: AU, CA, JP, KR, SK, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: CULTURING OF HEMATOPOIETIC STEM CELLS AND THEIR GENETIC ENGINEERING (57) Abstract Human hematopoietic stem cells may be grown in longterm culture, particularly co-cultures with an appropriate stromal cell line, particularly in the presence of leukemia inhibitory factor, by itself or in conjunction with other factors. The cells are found to retain their function as demonstrated by an <i>in vivo</i> T-cell assay and their ability to form colonies of other lineages in methylcellulose. Transfection of the cells may be achieved with a virus, where improved results are obtained in the presence of leukemia inhibitory factor and optionally other hematopoietic factors.		

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**CULTURING OF HEMATOPOIETIC STEM CELLS
AND THEIR GENETIC ENGINEERING**

INTRODUCTION

Technical Field

The field of this invention is expansion of human hematopoietic stem cells and their genetic modification.

5

Background

The availability of human hematopoietic stem cell compositions offers a plethora of opportunities for medicine. The hematopoietic stem cell is the progenitor for all of the blood cells, including leukocytes, including lymphocytic and myelomonocytic lineages, and erythrocytes, as well as other types of cells, such as osteoclasts. There is the further possibility, that the stem cell may also lead to stromal cells. These cells provide an enormous range of functions. None of these cells is believed to be capable of self-regeneration, so as to survive during the lifetime of the host. The stem cell is believed to be the only cell that is self-regenerating and maintains its pluripotent potential during the life of the host. Therefore, understanding the role of the stem cell, the manner in which it regenerates, and the manner in which it is programmed to produce the various lineages will provide opportunities for therapies for a wide variety of diseases.

25 The ability to obtain substantially homogeneous human stem cell compositions offers new approaches to bone

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marrow transplantation. Since there is evidence to suggest that the stem cell will not be malignant, by isolating human hematopoietic stem cells, one may avoid restoring malignant cells to a host after radiation or
5 chemotherapeutic treatment of cancer or other malignancy. The stem cells offer an entree to gene therapy, where the modification will survive the life of the host. In addition, by appropriate use of inducible promoters, one can provide that expression of various protein products
10 may be achieved at selected levels of differentiation or in selected lineages, or even in response to particular chemical clues, such as chemoattractants, particular ligands, and the like. Also, as there is better understanding of the manner in which stem cells are
15 directed to specific lineages, there will be the opportunity to produce in culture populations of particular lineages, such as megakaryocytes, subsets of T cells, monocytes, and the like.

An important aspect of this invention in the use of
20 stem cells will be the ability to expand the stem cells in culture. Growing stem cells is different from growing other cells, since in order to expand stem cells, one must not only provide for regeneration, but inhibit the loss of stem cells by differentiation. The manner in which
25 regeneration as against differentiation is regulated in bone marrow is not understood. Methods are therefore necessary which allow for long-term retention and expansion of stem cell cultures.

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Also, there has been no prior showing that one can genetically engineer a stem cell. The unique characteristics of the stem cell distinguish the stem cell from other cells which have been successfully genetically engineered. However, efforts to genetically engineer progeny cells frequently encounter lack of transmission of functional expression of the introduced construct in progeny cells, intermittent expression, and the like. Therefore, even where one has demonstrated the successful integration of the construct, there are instances encountered where subsequent growth of the progeny cells and their differentiation result in the failure of the construct to function.

15 Relevant Literature

U.S. Patent No. 5,061,620 describes a substantially homogeneous human hematopoietic stem cell composition and the manner of obtaining such composition. See also references cited therein. Stromal cell-associated hematopoiesis is described by Paul et al., Blood (1991), 77, 1723-1733. Murine leukemia inhibitory factor is taught to enhance retroviral-vector infection efficiency of hematopoietic progenitors by Fletcher et al., Blood (1990), 76, 1098-1103. Metcalf et al., Blood, (1990), 76, 50-56 describes the effect of injected leukemia inhibitory factor on hematopoietic and other tissues in mice. The in vitro effect of leukemia inhibitory factor on multipotential human hematopoietic is described by Vignani

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and McGlave, Blood (1991), 77, 263-270. Dick et al.,
Blood (1991), 78, 624-634 describe gene transfer into
normal human hematopoietic cells employing *in vitro* and *in*
vivo assays. The effect of leukemia inhibitory factor on
5 hematopoiesis is described by Metcalf, Phil. Trans. R.
Soc. Lond. (1990), B327, 99-109 and Leary et al., Blood
(1990), 75, 1960-1964.

A review of leukemia inhibitory factor (LIF) is found
in Metcalf, Intl. J. Cell Clon. (1991), 9, 95-108. A
10 description of the effect of LIF on embryonic stem cells
is provided by Nichols et al., Dev. (1990), 110,
1341-1348; Williams et al., Nature (1988), 336, 684-687
and Smith et al., Nature (1988), 336, 688-690.

15 SUMMARY OF THE INVENTION

Methods and compositions are provided for maintenance
and expansion of human hematopoietic stem cells in culture
by using leukemia inhibitory factor (LIF) under conditions
where human hematopoietic stem cells are able to survive
20 and to expand in culture. LIF may be used by itself or in
combination with other added hematopoietic cell factors in
an appropriate culture medium. A substantially
homogeneous human hematopoietic stem cell composition is
genetically modified using appropriate DNA constructs for
25 introduction into the stem cells and integration. The
genetic modification of the stem cells is demonstrated by
assays where long-term maintenance of expression of the

integrated gene is shown in a plurality of hematopoietic lineages, both lymphocytic and myelomonocytic.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

5 Substantially homogeneous human hematopoietic stem cells (hereinafter referred to as "hHSC") are maintained in long-term cultures and expanded in number in appropriate media comprising added leukemia inhibitory factor ("LIF"), optionally in conjunction with additional
10 added hematopoietic factors, under otherwise conventional conditions. The hHSC may be maintained in culture for long periods of time, as demonstrated by their capability to continually differentiate into multilineage progeny.

 The hHSC may be genetically modified by employing a
15 substantially homogeneous stem cell composition with a DNA construct providing a DNA sequence of interest. Particularly, a retroviral vector is employed for the introduction of the DNA construct into the hHSC host. The resulting cells may then be grown under conditions as
20 described for unmodified hHSC, whereby the modified hHSC may be expanded and used for a variety of purposes.

 The hHSC which are employed may be fresh, frozen, or have been subject to prior culture. They may be fetal, neonate, adult, obtained from fetal liver, bone marrow,
25 blood or any other conventional source. The manner in which the stem cells are separated from other cells, whether of the hematopoietic or of other lineage is not critical to this invention. Conveniently, the cells may

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be separated as described in U.S. Patent No. 5,061,620. As described, the substantially homogeneous composition of hHSC may be obtained by selective isolation of cells free of markers associated with differentiated cells, while displaying epitopic characteristics associated with the stem cells. The stem cells are characterized by both the presence of markers associated with specific epitopic sites identified by antibodies and the absence of certain markers as identified by the lack of binding of certain antibodies. At such time as a specific marker is identified for hHSC, binding of an antibody to such marker may provide the desired composition.

A large proportion of the differentiated cells may be removed by initially using a relatively crude separation, where major cell population lineages of the hematopoietic system, such as lymphocytic and myelomonocytic, are removed, as well as minor populations, such as megakaryocytic, mast cells, eosinophils and basophils. Usually, at least about 70 to 90 percent of the hematopoietic cells will be removed. If desired, a prior separation may be employed to remove erythrocytes, by employing ficoll-hypaque separation.

The gross separation may be achieved using magnetic beads, cytotoxic agents, affinity chromatography, panning, or the like. Antibodies which find use include antibodies of CD34, Class II HLA or other marker which allows for removal of most, if not all, mature cells, while being absent in hHSC.

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Concomitantly r subsequent to the gr ss s paration, which provides for positive selection, a negative selection may be carried out, where antibodies to specific markers present on dedicated cells are employed. For the
5 most part, these markers will include CD3⁺, CD7⁺, CD8⁺, CD10⁺, CD14⁺, CD15⁺, CD19⁺, CD20⁺, CD33⁺, preferably including at least CD3⁺, CD8⁺, CD10⁺, CD19⁺, CD20⁺, CD33⁺, normally including at least CD10⁺, CD19⁺, CD33⁺. The hematopoietic cell composition substantially depleted of dedicated cells
10 may then be further separated using a marker for Thy-1, whereby a substantially homogeneous stem cell population is achieved. Exemplary of this stem cell population is a population which is CD34⁺ Thy-1⁺, which approximates the substantially homogeneous stem cell composition.

15 The hHSC composition is characterized by being able to be maintained in culture for extended periods of time, being capable of selection and transfer to secondary and higher order cultures, and being capable of differentiating into the various lymphocytic and
20 myelomonocytic lineages, particularly B- and T-lymphocytes, monocytes, macrophages, neutrophils, erythrocytes, and the like.

A pluripotent human stem cells may be defined as follows: (1) gives rise to progeny all defined hemato-
25 lymphoid lineages; and (2) limiting numbers of cells are capable of fully reconstituting an seriously immunocompromised human host in all blood cell types and

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their progenitors, including the pluripotent hematopoietic stem cell by cell renewal.

The hHSC are then grown in culture in an appropriate nutrient medium, which medium may be a conditioned medium, a co-culture with an appropriate stromal cell line, or a medium comprising a synthetic combination of growth factors which are sufficient to maintain the growth of hematopoietic cells.

For conditioned media or co-cultures, various stromal cell lines may be used, since it is found that human stromal cell lines are not required. Thus, other stromal cell lines may be employed, such as rodentiae, particularly murine. A number of murine stromal cell lines are described in Whitlock et al., Cell (1987), 48, 1009-1021, AC6.21 being deposited at the ATCC as _____. Other stromal cell lines may be developed, if desired.

Various devices exist for the co-culture which allow for growth and maintenance of cells. Thus, devices employing crossed threads, membranes, controlled medium flow, and the like may be employed for the growth of the cells for removal of waste products, and replenishment of the various factors associated with cell growth.

Conveniently, tissue culture plates or flasks may be employed where confluent stromal cell layers may be maintained for extended periods of time without passage, but with changing of the tissue culture medium about every five to seven days.

The hHSC may be grown in co-culture by placing the hHSC onto the stromal cell lines, either directly or separated by a porous membrane. For example, for about 3×10^4 to 3×10^5 cells/ml are placed on a confluent stromal cell layer. The media employed in the co-culture may be any convenient growth medium, such as RPMI-1640, IMDM, etc. either individually or in combination, where appropriate antibiotics to prevent bacterial growth and other additives, such as pyruvate (0.1-5 mM), glutamine (0.5-5 mM), 2-mercaptoethanol ($1-10 \times 10^{-5}$ M) and from about 5-15%, preferably about 10% of serum, e.g. fetal calf serum.

In addition to the other additives, LIF is added in from about 1 ng/ml to 100 ng/ml, more usually 5 ng/ml to 30 ng/ml. Other factors may also be included, such as interleukins, colony stimulating factors, steel factor, or the like. Of particular interest in addition to LIF are IL-3, IL-6, and GM-CSF.

The factors which are employed may be naturally occurring or synthetic e.g. prepared recombinantly, and may be human or of other species, e.g. murine, preferably human.

The amount of the other factors will generally be in the range of about 1 ng/ml to 100 ng/ml. Generally, for IL-3, the concentration will be in the range of about 5 ng/ml to 50 ng/ml, more usually 5 ng/ml to 100 ng/ml; for IL-6, the concentration will be in the range of about 5 ng/ml to 50 ng/ml, more usually 5 ng/ml to 20 μ g/ml, and

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for GM-CSF, the concentration will generally be 5 ng/ml to 50 ng/ml, more usually 5 ng/ml to 20 ng/ml.

The LIF and other factors may be present only during the initial course of the stem cell growth and expansion, usually at least 24 hours, more usually at least about 48 hours or may be maintained during the course of the expansion. Thus, it is found that significant effect can be achieved by exposure of the hHSC initially in the growth medium, without maintaining the concentration during the entire course of the expansion.

For genetic modification, the cells may be grown for sufficient time to reach the desired population level. Usually, at least 1×10^4 hHSC cells will be present, preferably 1×10^5 cells. LIF will be present at least initially in the culture medium, usually for at least 12, more usually at least 24 h, where exposure to LIF and other growth factors may be substantially terminated. LIF and optionally the other growth factors may be maintained during the course of the growth of the cells. The cells will have been grown for at least 12, usually 24 hours, in the medium with the factors, before contact with the DNA construct. For genetic modification of the hHSC, usually a retroviral vector will be employed.

Various retroviral vectors may be employed for genetic modification. One will normally use combinations of retroviruses and an appropriate packaging line, where the capsid proteins will be functional for infecting human cells. Various amphotropic virus-producing cell lines are

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known, such as PA12 (Miller et al., Mol. Cell. Biol. 5
(1985), 431-437), PA317 (Miller et al., Mol. Cell. Biol.
6 (1986), 2895-2902) GRIP (Danos et al., PNAS 85 (1988),
6460-6464). Usually, the cells and virus will be
5 incubated for at least about 24 hours in the culture
medium. The cells are then allowed to grow in the culture
medium for at least two weeks, and may be allowed to grow
for five weeks or more, before analysis.

The constructs which will be employed will normally
10 include a marker, which allows for selection of cells into
which the DNA has been integrated, as against cells which
have not integrated the DNA construct. Various markers
exist, particularly antibiotic resistance markers, such as
resistance to G418, hygromycin, and the like. Less
15 conveniently, negative selection may be used, where the
marker is the HSV-tk gene, which will make the cells
sensitive to agents, such as acyclovir and gancyclovir.

The constructs can be prepared in a variety of
conventional ways. Numerous vectors are now available
20 which provide for the desired features, such as long
terminal repeats, marker genes, and restriction sites.
Thus, one may introduce the vector in an appropriate
plasmid and manipulate the vector by restriction,
insertion of the desired gene, with appropriate
25 transcriptional and translational initiation and
termination regions, and then introduce the plasmid into
an appropriate packaging host. Thus, at each of the
manipulations, one may grow the plasmid in an appropriate

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prokaryotic host, analyze the construct to ensure that the desired construct has been obtained, and then subject the construct to further manipulation. When completed, the plasmid or excised virus may then be introduced into the packaging host for packaging and isolation of virus particles for use in the genetic modification.

The introduction of DNA can be used for a wide variety of purposes, such as gene therapy, introduction of novel capabilities into the hHSC, direct dedication to a particular lineage or subset of such lineage, enhancement of maturation to a particular lineage or subset, or the like. In view of the major role hematopoietic cells play in the functioning of the human host, their wide-spread presence, and their varied capabilities, the hHSC have great therapeutic potential.

There are many genetic diseases specific for hematopoietic cells, including sickle cell anemia, β -thalassemia, thrombocytopenia, hemophilia, combined immunodeficiency, and most leukemias. For the most part, these diseases may be treated by homologous recombination, where at least one copy of the defective gene may be modified to the wild-type or a functioning gene. For the most part, it will not be necessary to correct both copies, usually correcting one copy sufficing to provide for therapeutic treatment. There are numerous descriptions of methods of homologous recombination in the literature, see for example, Mansour, et al., Nature

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(1988) 336, 348-352 and Schwartzberg, et al., PNAS USA
(1990) 87, 3210-3214.

Alternatively, one may use the hHSC and their progeny as carriers for the production of a wide variety of products, where the host is genetically deficient or as a result of a subsequent disease has become genetically deficient. Genetic diseases involving lack of a particular natural product include muscular dystrophy, where there is a lack of dystrophin, cystic fibrosis, Alzheimer's disease, Gaucher disease, etc. In those instances where a particular polymorphic region of a polymorphic protein such as a T-cell receptor, major histocompatibility complex antigen, or immunoglobulin subunit is involved with susceptibility to a particular disease, e.g. an autoimmune disease, the particular exon may be "knocked out" by homologous recombination, so as to provide hematopoietic cells which will not be responsive to the disease.

In other situations, such as diabetes, where cells have been destroyed as a result of autoimmunity, the hHSC may be modified to provide for cells which will respond to the need for secretion of insulin, where appropriate enhancers and promoters may be employed, so as to have the insulin production regulated in analogous manner to the regulation in the islets of Langerhans. This may involve the expression of the insulin receptor in an appropriate hematopoietic lineage.

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Also, multiple drug resistance gene(s), e.g. pgp-1, may be introduced to protect the cells against cytotoxic drugs, transcription of ribozymes to protect against viral infection, or expression of various protein products to
5 inhibit viral replication intracellularly, e.g. the tat gene with HIV.

Modified stem cells may also be found for use in the treatment of aging, autoimmune diseases, hematopoietic disorders, and viral infections.

10 In many situations the therapy involves removal of bone marrow or other source of stem cells from a human host, isolating the stem cells from the source and expanding the stem cells. Meanwhile, the host may be treated to substantially or complete ablate native
15 hematopoietic capability. The stem cells may be modified during this period of time, so as to provide for stem cells having the desired genetic modification. After completion of the treatment of the host, the modified stem cells may then be restored to the host to provide for the
20 new capability. If necessary, the process may be repeated to ensure the substantial absence of the original stem cells and the substantial population of the modified stem cells.

To prove that one has the modified stem cells,
25 various techniques may be employed. The genome of the cells may be restricted and used with or without amplification. The polymerase chain reaction, gel electrophoresis, restriction analysis, South rn, Northern,

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and Western blots may be employed, sequencing, or the like, may all be employed with advantage. In addition, the cells may be grown under various conditions to ensure that the cells are capable of maturation to all of the hematopoietic lineages while maintaining the capability, as appropriate, of the introduced DNA. Various tests *in vitro* and *in vivo* may be employed to ensure that the pluripotent capability of the stem cells has been maintained.

10 To demonstrate differentiation to T cells, one may isolate fetal thymus and culture the thymus for from 4-7 days at about 25°C, so as to substantially deplete the lymphoid population of the fetal thymus. The cells to be tested are then microinjected into the thymus tissue, where the HLA of the population which is injected is mismatched with the HLA of the thymus cells. The thymus tissue may then be transplanted into a scid/scid mouse as described in EPA 0 322 240, where the thymus is conveniently transplanted into the kidney capsule.

20 For erythrocytes, one may use conventional techniques to identify BFU-E units for example methylcellulose culture (Metcalf, In: Recent Results in Cancer Res. (1977), 61. Springer-Verlag, Berlin, pp. 1-227) demonstrating that the cells are capable of developing the erythroid lineage.

25 In identifying myeloid and B cell capability, conveniently, the population to be tested is introduced first into a hydrocortison containing culture and allowed

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to grow for six weeks in such culture. The medium employed will comprise a 50:50 mixture of RPMI1640 and IMDM containing 10% FCS, 10% horse serum, 50 μ g/ml streptomycin/penicillin, glutamine and 5×10^{-7} M hydrocortisone. During the six-week period, it will be anticipated that in the absence of progenitor cells, all of the mature cells would die. If at the end of six weeks, myeloid cells are still observed, one may conclude that there is a progenitor cell which is providing for the continuous differentiation to myeloid cells.

At this time, one may then change the medium, so that the medium now lacks hydrocortisone, to encourage the growth of B cells. By waiting 3-4 weeks and demonstrating the presence of B cells by FACS analysis or other analytical procedure, one may conclude that the progenitor cells which previously were capable of producing myeloid cells are also capable of producing B cells.

Human hematopoietic cells grown in the presence of hydrocortisone can be maintained for at least four months. Similarly, human hematopoietic cell cultures can be grown in the absence of hydrocortisone for at least four months, which cultures will contain B lymphocytes as well as myelomonocytic cells. One may then sort the cell cultures for identification of hHSC.

The stem cells may be administered in any physiologically acceptable medium, normally intravascularly, although they may also be introduced into bone or other convenient site, where the cells may find an

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appropriate site for regeneration and differentiation. Usually, at least 1×10^5 cells will be administered, preferably 1×10^6 or more. The cells may be introduced by injection, catheter, or the like. If desired, depending upon the purpose of the introduction of the cells, factors may also be included, such as the interleukins, e.g. IL-2, IL-3, IL-6, and IL-11, as well as the other interleukins, the colony stimulating factors, such as G-, M- and GM-CSF, interferons, e.g. γ -interferon, erythropoietin, etc. The amount of these various factors will depend upon the purpose of the administration of the cells, the particular needs of the patient, and will normally be determined empirically.

The stem cell compositions which are employed will generally have fewer than 5% of lineage committed cells and will be capable of cell-free generation in a co-culture medium and differentiation to members of the lymphoid and myelomonocytic hematopoietic lineages. They will generally have at least 80% of the cells characterized by being human, hematopoietic, and being CD34⁺ 10⁻ 19⁻ 33⁻ and Thy-1⁺. In addition, they may when stained with rhodamine, be either rhodamine high or rhodamine low or a combination thereof. Preferably, the cells will be rhodamine low. See Spangrude, Immunol. Today (1989), 344-350. The cells may be frozen at liquid nitrogen temperatures and stored for long periods of time, being thawed and capable of being reused. The cells will usually be stored in a 10% DMSO, 50% FCS, 40% RPMI1640

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medium. Once thawed, the cells may be expanded by use of growth factors and/or stromal cells associated with stem cell proliferation and differentiation.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Materials and Methods

Antibodies. The antibodies to CD34 were obtained from I.D. Bernstein (Andrews et al., Blood (1986), 68, 1030); the antibody for Thy-1 was obtained from Fabre (Dalchau and Fabre, J. Exp. Med. (1979), 149, 576). The antibodies for CD34 were detected using the appropriate anti-Ig conjugated to fluorescein, phycoerythrin, or Texas red (Cal Tag) or bound to magnetic beads (Applied Immune Sciences [AIS]) and separated magnetically. The Thy-1 antibody was a fluorescein, phycoerythrin or biotin conjugate, where the biotin conjugate was detected with Texas red-avidin (Cal Tag).

Fluorescence Activated Cell Sorter (FACS) Analysis and Sorting. A Becton-Dickinson FACS modified as described (Parks and Herzenberg, Meth. Enzymol. (1984), 108, 197) was employed. The dual laser instrument allows for four fluorescent parameters and two light scatter parameters to be recorded for each analyzed cell. Residual erythrocytes and dead cells and debris were excluded from analysis by light scattering gating and propidium iodide (PI) staining or by scattering alone in

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four color analyses. Compensation for spatial verlaps of fluoresc in and phycoerytherin and fluorescein and propidium iodide was adjusted electronically.

For cell sorting, the stained samples were maintained
5 at 4°C throughout the sorting procedure. Sorted drops were collected in RPMI1640 containing 10% fetal calf serum. Following isolation of a cell population by FACS, the sample was diluted 1:1 in HBSS, centrifuged for 10 min at a rcf of 200 and resuspended in 50 or 100 µl of HBSS
10 for hemocytometer counting.

The culture assays were performed as follows: AC6.21
confluent stromal cell layers were maintained for up to 3-4 weeks without passage by changing of the tissue culture medium every 5-7 days. To passage, the stomal
15 cell layers were washed three times with serum-free medium, then overlaid with 2.5 ml (T-25 flask) of 0.5 mg/ml collagenase-dispase (Boehringer-Mannheim, Indianapolis, IN) in serum-free medium. The cultures were allowed to incubate 15-30 min at 37°C; then the cells in
20 the enzyme-containing medium were collected in RPMI1640 medium with serum added. The stromal cells were suspended by pipetting with a Pasteur pipet, then cultured directly at one-fifth to one-fiftieth of the original cell concentration. Generally, confluent stromal layers
25 subcultured at 1:10 reached confluency again at after 5-7 days. Subclones were obtained by limiting dilution culture from 30 to 0.3 cells per well.

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Cell suspensions of human fetal bone marrow were prepared from long bones of fetuses from 16-20 week gestation. The bones are split lengthwise and the medullary cavity is scraped with a scalpel blade. The bones are then placed in a 1 mg/ml solution of collagenase-dispase in RPMI1640. The bones are incubated for 30 min at 37°C, after which time the medullary cavity is flushed with media (RPMI1640 with pen/strep, 2-ME and 5% FCS) to remove hematopoietic cells. Alternatively, bone marrow may be flushed from the marrow cavity without the collagenase-dispase treatment.

Cell suspensions are prepared from livers of 16-20 week gestation fetuses. The liver is minced and then pipetted to release cells. The cell suspension is then placed on Ficoll gradient to remove hepatocytes, red blood cells and debris. The hematopoietic cells are then harvested.

Adult bone marrow is obtained from aspirates, which are treated to remove red blood cells before use.

Bulk cultures are obtained by placing the human cells on the previously established confluent layer of mouse stromal cell lines. From 3×10^4 to 2×10^5 cells per ml are placed on the stromal cells in either T-25 flasks or six-well plates, by addition of 3 ml to each well of a six-well plate or 5 ml to a T-25 flask. A 50:50 mixture of RPMI1640 and IMDM containing 50 U/ml penicillin/50 μ g/ml streptomycin, 1 mM sodium pyruvate, 2 mM glutamine, 5×10^{-5} M 2-mercaptoethanol and 10% fetal calf serum is employed.

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For Dexter-type conditions, IMDM containing 50 U/ml penicillin/50 µg/ml streptomycin, 1 mM sodium pyruvate, 2 mM glutamine, 10% fetal calf serum, 20% horse serum and 10^{-6} M hydrocortisone sodium succinate is employed. Bone marrow cells grown in the Dexter-type medium give rise only to myeloid differentiation. Cultures were established with whole-cell populations or cells fractionated by their expression of cell surface antigens (CD34, Thy-1).

One can determine the frequency of cells in the starting population which grow under the above-defined conditions. The frequency is determined by the cell number at which 37% of the wells show no growth.

The Effect of Growth Factors on Cell Growth. Into 10 ml of long-term culture medium was added 100 µl (about 100 cells) per well in a 96 well plate, where each of the wells had a confluent stromal monolayer of AC6.21. One-half of the medium was replaced with fresh long-term culture medium every week. Different factors were added, individually or in combination, where the amount added was the optimum concentration to maximize the number of cells obtained under the above conditions in 35 days. The factors employed were IL-3, IL-6, GM-CSF, steel factor (SL) and LIF. These factors were obtained as lyophilized recombinant proteins (powder) from R&D Systems (Minneapolis). At the end of various time periods, 10-15 wells were screened by FACS analysis and the total number

of cells counted. The following table (Table I) indicates the results.

TABLE I
CELL GROWTH

	<u>Treatments</u>	<u>Time</u>					2	3
		2 weeks	3 weeks	4 weeks	5 weeks	6 weeks	7 weeks	
5	None						>2,000	
	3 + 6						>2,000	
	GM						>5,000	
	3 + 6 + GM						>5,000	
	SL						>2,000	
10	LIF							100,000
	LIF + 3 + 6				100,000			
	LIF + 3 + 6 + GM		10,000	200,000	200,000			
	LIF + 3 + 6 + GM + SL		10,000	200,000	200,000			
15								

Note: There is no difference in the cell growth between the same treatment with or without virus infection.

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In the next study, the presence of cells having various markers was determined. CD7 is a T-cell marker and not unexpectedly, T-cells were not observed, since there is no exposure to a thymic environment. CD15
5 indicates monocytes and granulocytes, CD19 indicates B-cells and CD33 indicates myeloid cells. By frequency is intended the number of wells in which cells carrying the markers were observed of 15 wells (Table II).

TABLE II
FACS ANALYSIS OF 4 WEEKS CULTURES

5

Markers	Treatments			
	LIF + 3 + 6 + GM + SL	LIF + 3 + 6 + GM	LIF + 3 + 6 + GM + virus	
CD 19	11/15	12/15	11/15	
CD 15	15/15	15/15	15/15	
CD 33	15/15	15/15	15/15	
CD 7	0/15	0/15	0/15	

10

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In the next study, a FACS analysis was made of the CD34, Thy-1 populations after 5 weeks in the above culture. The next table shows the percentage of CD34 Thy-1⁺ populations after 5 weeks and the number of wells which were positive of the 15 wells analyzed by FACS (Table III).

TABLE III
FACS ANALYSIS FOR THE CD34⁺ THY-1⁺
POPULATIONS IN THE 5 WEEKS IN VITRO CULTURES

	Treatments	CD34 ⁺ Thy-1 ⁺ populations	
		Frequency	Range of the Percentage
5	LIF + 3 + 6	13/15	5.56% - 16.27%
			average 9.2%
	LIF + 3 + 6 + GM	11/15	5.9% - 25.3%
			average 10.5%
10	LIF + 3 + 6 + GM + SL	13/15	5.65% - 21.56%
			average 11.3%
	LIF + 3 + 6 + virus	12/15	5.5% - 18.15%
			average 9.8%
15			

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In the next study, the above study was repeated, except that the culture was maintained for seven weeks. The following table indicates the results (Table IV).

TABLE IV
FACS ANALYSIS OF 7 WEEKS IN VITRO CULTURES

5	CD34 ⁺ Thy-1 ⁺	AC6	GM	3 + 6	3 + 6 + GM	LIF
	Frequency*	0/6	0/9	0/15	0/15	12/15
	Range of Percentage**					1.5% - 13%

* Greater than 1% will be scored as positive.

10 ** The average is 4.5%, and the median is 5%.

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From the above results the following conclusions are that the stem cell populations in the *in vitro* cultures have the right surface markers and the right cell size and granularity (Paint-a-Gate).

5 To further demonstrate the activity of the CD34 Thy-1 population after *in vitro* culture, secondary *in vitro* cultures were prepared. The cells from 50 wells of the 24 hour treatment with LIF, IL-3 and IL-6 after 5 weeks were sorted for the CD34 Thy-1 populations. The total cells
10 were about 5×10^6 . After sorting, about 5×10^5 of CD34 Thy-1 cells were obtained. These were promptly introduced into wells comprising confluent cultures of AC6.21 with long-term culture medium at about 100 cells per well. The following table indicates the results.

TABLE V
FACS ANALYSIS FOR THE CD34⁺ THY-1⁺
POPULATIONS IN THE 5 WEEKS IN VITRO CULTURES

5

Treatments	CD34 ⁺ Thy-1 ⁺ populations	
	Frequency	Range of the Percentage
LIF + 3 + 6	13/15	5.56% - 16.27% average 9.2%
10 LIF + 3 + 6 + GM	11/15	5.9% - 25.3% average 10.5%
LIF + 3 + 6 + GM + SL	13/15	5.65% - 21.56% average 11.3%
15 LIF + 3 + 6 + virus	12/15	5.5% - 18.15% average 9.8%

TABLE V (continued)
FACS ANALYSIS FOR THE CD34⁺ THY-1⁺
POPULATIONS IN THE 5 WEEKS SECONDARY CULTURES

<u>Treatments</u>	<u>CD34⁺ Thy-1⁺</u>	
	<u>Frequency</u>	<u>Range of the Percentage</u>
LIF + 3 + 6	6/15	4.1% - 13%
LIF + 3 + 6 + GM	6/15	4.3% - 15%
LIF + 3 + 6 + GM + SL	8/15	4.9% - 16%

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Infection of CD34⁺ Thy-1⁺ Population. The amphotropic retroviral vector ΔMo+PyF101-μ^R was employed. Valerio et al., Gene (1989), 84, 419-427; and Bambesechem et al., J. Exp. Med. (1990), 172, 729-736. The infection was performed by introducing approximately 10⁴ CD34 Thy-1⁺ cells and 1x10⁶ CFU of the virus in 1 ml of long-term culture medium ("LTCM") comprising 10 ng/ml each of LIF, IL-3, IL-6, and GM-CSF. The cell mixture was maintained for 24 hours, and the medium diluted to 10 ml with LTCM and 100 μl of the medium introduced into wells in which confluent layers of the AC6.21 stromal line was present. The cells from 5-15 wells, each of the cells with and without virus, were then introduced into a methylcellulose culture and maintained for 2 weeks. At the end of this time, all cells were collected from each methylcellulose culture and analyzed by DNA PCR for the neo gene.

In a second series of experiments, to the methylcellulose culture was added G418 at 1 mg/ml and cells in 5 wells, which had not been contacted with the virus, and cells in 20 wells which had been contacted with the virus, were prepared. The cultures were maintained for 2 weeks, after which all of the cells from each culture were isolated and subject to the same analysis for the neo gene. Also, the cells were analyzed for the presence of the polyoma enhancer sequence.

The primers employed are as follows:

CA TCGCATGAG CGAGCACGTA (SEQ. ID NO:1) Neo-1

CGATGCCTGC TTGCCGAATA TCATG (SEQ. ID NO:2) Neo-2

CTAGACTGG CCGTGCGACA TCCTCT (SEQ. ID NO:3) PyF101-3

5 CAAT CATTACTATG ACAACAGTCT AG (SEQ. ID NO:4) PyF101-4

The anticipated fragment with primers for the neo gene was 180 bp. Samples from cells which had not been selected with G418, and which were not exposed to the virus showed no band, while 3 samples from cells which had been exposed to the virus were positive. The negative and positive controls for PCR were as expected. A 2% agarose gel was employed for electrophoretic separation.

In the electrophoresis for cells which had been exposed to the virus, the 3 control samples of cells which had not been exposed to the virus were all negative, while cells from 12 wells, where the cells had been exposed to the virus were all positive.

To further establish the absence of any artifacts, the PCR products were digested with restriction enzyme SphI which provides 120 bp and 60 bp fragments, or NcoI which provides 150 and 30 bp fragments. The resulting gels provided the anticipated bands; where cells which had not been infected with virus were negative, while cells which had been infected with virus showed 2 smaller bands than the original band.

In the next study, the uninfected cells and infected cells were studied to determine their ability to provide long-term T-cell reconstitution in the thymus of the SCID-

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hu/thymus model, where fetal thymus is introduced into the kidney capsule of a C.B.17 scid/scid mouse. The cells which are employed for culture differ in HLA from the cells of the thymus. (See PCT/US91/02373 for a description of the test procedure.) 10⁴ CD34 Thy-1 cells from five-week cultures, where the cells had originally been exposed for 24 hours to LIF plus IL-3 plus IL-6 or these factors plus virus as described previously and allowed to grow for five weeks, were employed. At the end of this time, the cells were sorted for CD34 Thy-1 for injection into the thymus. FACS analysis showed the presence of T-cells observed by markers for the surface membrane proteins CD3, CD4 and CD8 in conjunction with a marker for the HLA of the donor cells in two or three studies. Similarly, when the same T-cell markers were employed as against an antibody for all human cells, in the same two or three experiments, a large population of T-cells were observed.

The cells in the thymus were isolated and subjected to DNA PCR analysis. Where the primers employed were for the neo gene, employing a total of 10⁶ cells from the thymus, a control mouse gave a negative result, while a positive result is observed under the following conditions: 3 months after injection CD34⁺ Thy-1⁺ positive cells from a secondary culture at 10⁴ cells/thymus; 2.5 months after injection of CD34⁺ Thy-1⁺ positive cells from a primary culture, 2x10³ cells/thymus; 5 months after injection of CD34⁺ Thy-1⁺ cells from a primary culture, at

10⁴ cells/thymus. Analogous results were observed for the polyoma enhancer.

It is evident from the above results, that pluripotent hematopoietic stem cells can be grown for extended periods of time, substantially expanded, in culture, while retaining pluripotency. Thus, stem cells may be obtained from a wide variety of sources, fetal or adult, bone marrow or blood, and grown, so as to have an expanded source of the cells. Furthermore, cells may be infected with appropriate retroviruses, where the cells will integrate the retroviral construct with functional expression of a gene contained within the construct, where in cells after differentiation and maturation, the gene is retained and will be expressed.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: SyStemix, Inc.
- (ii) TITLE OF INVENTION: Culturing of hematopoietic stem cells and their genetic engineering.
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Bertram I. Rowland
 - (B) STREET: 4 Embarcadero Center, Suite 3400
 - (C) CITY: San Francisco
 - (D) STATE: California
 - (E) COUNTRY: USA
 - (F) ZIP: 94111
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: PCT/US93/
 - (B) FILING DATE: 03-MAR-1993
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Rowland, Bertram I
 - (B) REGISTRATION NUMBER: 20,015
 - (C) REFERENCE/DOCKET NUMBER: FP-55698/BIR
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (415) 781-1989
 - (B) TELEFAX: (415) 398-3249

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CATCGCATGA GCGAGCACGT A

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGATGCCTGC TTGCCGAATA TCATG

25

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTAGACTGGC CGTGCGACAT CCTCT

25

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CAATCATTAC TATGACAACA GTCTAG

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WHAT IS CLAIMED IS:

1. In a method for growing human hematopoietic stem cells in a culture medium, the improvement which
5 comprises:

contacting said stem cells with a medium comprising at least about 10 ng/ml of leukemia inhibitory factor for at least 12 h; and

growing said stem cells in a medium capable of
10 supporting the growth of said stem cells.

2. A method according to Claim 1, wherein said leukemia inhibitory factor is present in from about 2 to 20 ng/ml.

15

3. A method according to Claim 1, wherein said contacting is with at least one of IL-3, IL-6, GM-CSF, or steel factor in a least 1 ng/ml.

20

4. A method according to Claim 1, wherein said growing is in the presence of conditioned medium from a stromal cell line.

5. A method according to Claim 4, wherein said
25 stromal cell line is present in said medium.

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6. In a method for growing human hematopoietic stem cells in a culture medium, the improvement which comprises:

contacting said stem cells with a medium comprising
5 from about 2 to 20 ng/ml of leukemia inhibitory factor and at least one of IL-3, IL-6, GM-CSF, or steel factor in at least 1 ng/ml for at least about 12 h; and

growing said stem cells in a medium capable of supporting the growth of said stem cells.

10

7. A method according to Claim 6, wherein said growing is in the presence of conditioned medium from a stromal cell line.

15

8. A method according to Claim 7, wherein said stromal cell line is present in said medium.

20

9. A method according to Claim 6, wherein said leukemia inhibitory factor is maintained in said growth supporting medium.

10. A method for transfecting human hematopoietic stem cells, said method comprising:

contacting human hematopoietic stem cells
25 substantially free of dedicated cells with a retroviral vector tropic for human cells and comprising a DNA sequence of interest capable of transcription in human hematopoietic cells in a medium comprising at least

10 ng/ml of leukemia inhibitory factor for a time sufficient for said virus to enter said stem cells.

11. A method according to Claim 10, wherein said
5 medium further comprises at least one of IL-3, IL-6, GM-CSF, or steel factor in a least 1 ng/ml.

12. A method according to Claim 10, wherein said DNA
10 sequence is a gene capable of transcription in human hematopoietic cells.

13. A method according to Claim 10, further
comprising growing said hematopoietic stem cells
comprising said DNA sequence in culture in a medium
15 capable of supporting the growth of stem cells.

14. A method according to Claim 13, wherein said
growth supporting medium is a conditioned medium from a
stromal cell line.

20

15. A method according to Claim 14, wherein said
stromal cell line is present in said medium.

16. A method according to Claim 15, wherein said
25 leukemia inhibitory factor is maintained in said growth supporting medium.

17. A human hematopoietic stem cell composition substantially free of dedicated hematopoietic cells comprising a DNA sequence of interest resulting from integration of DNA.

5

18. A human hematopoietic stem cell according to Claim 17, wherein said DNA sequence of interest is a gene capable of transcription in a hematopoietic cell.

10

19. A human hematopoietic stem cell according to Claim 18, wherein said gene is a G418 resistance gene.

20. A human hematopoietic cell descended from an human hematopoietic stem cell according to Claim 17.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/01852

A. CLASSIFICATION OF SUBJECT MATTER

IPC(S) : C12N 5/06, 15/64

US CL : 435/172.3, 240.2; 935/52

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 240.2; 935/52

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSYS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
<u>X</u> Y	BLOOD, Volume 77, No. 2, issued 15 January 1991, C. Verfaillie et al., "Leukemia Inhibitory Factor/Human Interleukin for DA Cells: A Growth Factor That Stimulates the In Vitro Development of Multipotential Human Hematopoietic Progenitors", pages 263-270, see entire document.	<u>1-3, 6, 9</u> 1-20
Y	NATURE, Volume 336, issued 15 December 1988, J.-F. Moreau et al., "Leukaemia inhibitory factor is identical to the myeloid growth factor human interleukin for DA cells", pages 690-692, see entire document.	1-20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

16 April 1993

Date of mailing of the international search report

06 MAY 1993

Name and mailing address of the ISA/US
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	R. I. FRESHNEY, "CULTURE OF ANIMAL CELLS: A MANUAL OF BASIC TECHNIQUES", published 1987 by Alan R. Liss, Inc. (N.Y.), see pages 60-61, 139-140.	1-20
Y	JOURNAL OF EXPERIMENTAL MEDICINE, Volume 172, issued September 1990, V. W. van Beusechem et al., "Expression of Human Adenosine Deaminase in Mice Transplanted with Hemopoietic Stem Cells Infected with Amphotropic Retroviruses", pages 729-736, see entire document.	10-20
Y	BLOOD, Volume 76, No. 6, issued 15 September 1990, F. A. Fletcher et al., "Murine Leukemia Inhibitory Factor Enhances Retroviral-Vector Infection Efficiency of Hematopoietic Progenitors", pages 1098-1103, see especially page 1099.	10-20
Y	GENE, Volume 84, issued 1989, D. Valerio, "Retrovirus-mediated gene transfer into embryonal carcinoma and hemopoietic stem cells: expression from a hybrid long terminal repeat", pages 419-427, see entire document.	19